



Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatoxMolecular and physiological responses to long-term sublethal ammonia exposure in Atlantic salmon (*Salmo salar*)Jelena Kolarevic^{a,*}, Harald Takle^{b,e}, Olga Felip^c, Elisabeth Ytteborg^b, Roger Selset^a, Christopher M. Good^d, Grete Baeverfjord^a, Torbjørn Åsgård^a, Bendik Fyhn Terjesen^a^a Nofima, NO-6600 Sunndalsøra, Norway^b Nofima, NO-1432 Ås, Norway^c University of Barcelona, Department of Animal Physiology, Avda Diagonal 645, Barcelona, Spain^d The Conservation Fund's Freshwater Institute, 1098 Turner Road, Shepherdstown, WV, USA^e AVS Chile S.A., Casillas 300, Pto. Vares, Chile

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ABSTRACT

The objective of this study was to determine the underlying physiological and molecular responses to long-term sublethal ammonia exposure in Atlantic salmon (*Salmo salar*) parr. Previous studies have predominantly focused on mechanisms during acute, short-term exposure. For that purpose Atlantic salmon parr were exposed to four ammonia concentrations between 4 and 1800 $\mu\text{mol l}^{-1}$ total ammonia nitrogen (TAN), and subjected to two feeding regimes for 15 weeks. Elevated environmental ammonia and full feeding strength caused an initial increase in plasma ammonia levels ($[T_{\text{amm}}]$) after 22 days of exposure, which thereafter declined and remained similar to the control animals towards the end of the study. On the other hand, a progressive decrease in plasma urea levels was evident throughout the entire exposure period and depended on the concentration of environmental ammonia, with the largest decrease in urea levels observed at the highest ammonia concentrations (1700 and 1800 $\mu\text{mol l}^{-1}$ TAN). We hypothesized that the successful adaptation to long-term elevated ammonia levels would involve an increased capacity for carrier-facilitated branchial excretion. This hypothesis was strengthened by the first evidence of an up-regulation of branchial transcription of the genes encoding the Rhesus (Rh) glycoproteins, Rhcg1 and Rhcg2, urea transporter (UT) and aquaporin 3a (Aqp3a), during long-term exposure. Of the Rhesus glycoprotein (Rh) mRNAs, *Rhcg1* was up-regulated at all tested ammonia levels, while *Rhcg2* showed a concentration-sensitive increase. Increased transcription levels of V-type H^{+} -ATPase (H^{+} -ATPase) were observed at the highest ammonia concentrations (1700 and 1800 $\mu\text{mol l}^{-1}$ TAN) and coincided with an up-regulation of *Rhcg2* at these concentrations. Transcription of *UT* and *Aqp3a* was increased after 15 weeks of exposure to low ammonia levels (470 and 480 $\mu\text{mol l}^{-1}$ TAN). A significant increase in brain glutamine (Gln) concentration was observed for full fed Atlantic salmon after 22 days and in fish with restricted feeding after 105 days of exposure to 1800 and 1700 $\mu\text{mol l}^{-1}$ TAN, respectively, without any concomitant decrease in brain glutamate (Glu) concentrations. These results suggest that Gln synthesis is an ammonia detoxifying strategy employed in the brain of Atlantic salmon parr during long-term sublethal ammonia exposure. Full feed strength had an additive effect on plasma $[T_{\text{amm}}]$, while the restricted feeding regime postponed the majority of the observed physiological and molecular responses. In conclusion, Atlantic salmon parr adapts to the long-term sublethal ammonia concentrations with increased branchial transcription levels of ammonia and urea transporting proteins and ammonia detoxification in the brain.

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Abbreviations: TAN, total ammonia nitrogen; plasma $[T_{\text{amm}}]$, plasma ammonia levels; Rh, Rhesus glycoprotein; UT, urea transporter; Aqp, aquaporin; H^{+} -ATPase, V-type H^{+} -ATPase; mRNA, messenger RNA; Gln, glutamine; Glu, glutamate; Gsase, glutamine synthetase; HEA, high environmental ammonia; FAA, free amino acids; Asp, aspartic acid; Thr, threonine; Ala, alanine; Lys, lysine; HEA, chronic high environmental ammonia; P_{NH_3} , partial pressure of NH_3 .

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1. Introduction

Ammonia toxicity is well documented for a number of fish species (Ip et al., 2001a; Twitchen and Eddy, 1994; USEPA, 1998). As a final product of protein catabolism in most teleosts, ammonia is produced and excreted into the surrounding environment across the gills (Randall and Tsui, 2002; Randall and Wright, 1987). The increase in anthropogenic born ammonia in natural waters and occurrence of elevated ammonia in aquacultural systems can

hinder ammonia excretion or cause net uptake of ammonia from the environment with adverse effects on fish performance (growth, feed utilization) and welfare (Eddy, 2005; Randall and Tsui, 2002). Fish use a number of different strategies to ameliorate ammonia toxicity such as decreased ammonia production, conversion of ammonia to less toxic compounds, or increased ammonia excretion through the gills (reviewed in Randall and Tsui, 2002).

Increased glutamine formation, catalyzed by glutamine synthetase (GSase), is an important ammonia detoxification strategy observed in several species (Anderson et al., 2002; Ip et al., 2001b; Jow et al., 1999; Wright et al., 2007). GSase activity and Gln levels are typically high in ammonia-sensitive neural tissues in response to elevated environmental ammonia concentrations (Peng et al., 1998; Vedel et al., 1998; Wang and Walsh, 2000) and during postprandial plasma ammonia surges (Wicks and Randall, 2002a; Zimmer et al., 2010). Removal of NH_4^+ prevents the depolarization effect of this ion on neural membranes and excessive activation of NMDA type Glu receptors that have been proposed as causes of ammonia toxicity in several fish species (McKenzie et al., 2003; Randall and Tsui, 2002), although new evidence indicates that ammonia toxicity may be caused by impaired K^+ balance in the nervous system (Wilkie et al., 2011).

Insights into mechanisms of ammonia excretion in fish have been broadened by the discovery of the ammonia transporting function of Rhesus (Rh) proteins (Nakada et al., 2007). These initial findings were confirmed for a number of species (Nawata et al., 2007; Shih et al., 2008) and a new model for ammonia excretion in freshwater fish was proposed by Wright and Wood (2009). According to this model, an apical ' $\text{Na}^+/\text{NH}_4^+$ exchange complex' consisting of several membrane transporters, facilitates branchial ammonia excretion that is loosely or indirectly coupled with Na^+ uptake and acid excretion. Briefly, ammonia is transported from the blood, through gills and into the surrounding water via a series of Rh proteins, while acidification of the gill boundary layer via V-type H^+ -ATPase, H^+/Na^+ exchanger (NHE-2 or NHE-3) or hydration of CO_2 maintains a favourable partial pressure gradient for NH_3 and provides an electrochemical gradient for Na^+ uptake in the gills. This system is up-regulated at the mRNA level in response to a short-term exposure to high environmental ammonia (HEA) (Hung et al., 2007; Nawata et al., 2007), postprandial ammonia loading (Zimmer et al., 2010), and ammonium salt infusions (Nawata and Wood, 2009).

Adult ammonotelic teleosts maintain low levels of circulating urea produced via uricolysis and arginolysis which is excreted to the surrounding environment (Anderson, 2001; McDonald et al., 2006). Branchial expression of urea transporter (UT) mRNA was documented for a number of teleosts (Walsh et al., 2001) and the protein function in urea excretion was evident across the basolateral membranes of trout gill epithelium (McDonald and Wood, 2004).

Urea and ammonia may also be excreted across the fish gills through aquaporins (Aqp) (Cutler et al., 2007; Cutler and Cramb, 2002; McDonald et al., 2006; Weihrauch et al., 2009). Aqp represent a superfamily of major intrinsic proteins (MIPs) that facilitate passive transport of water molecules across the cellular membrane, while some Aqp also permeate non-ionic compounds such as urea, glycerol or ammonia and are termed as aquaglyceroporins (Cerdà and Finn, 2010; Tingaud-Sequeira et al., 2010). Using functional expression in *Xenopus* oocytes it was recently shown that Aqp3 in killifish (*Fundulus heteroclitus*) and European eel (*Anguilla anguilla*) transports urea and glycerol (MacIver et al., 2009; Tingaud-Sequeira et al., 2009) while Aqp3 might also be involved in ammonia excretion across the gills (Cutler et al., 2007).

The majority of ammonia exposure studies done to date on teleosts are acute studies. In contrast, mechanisms involved in ammonia handling during chronic long-term ammonia exposure,

have received less attention, especially in the case of Atlantic salmon in freshwater. In addition, most of the reported chronic ammonia exposure studies have aimed to determine the thresholds for chronic toxic effects in different species, resulting in mortality, while only a few have investigated the effect of environmentally relevant ammonia concentrations where mortality does not occur (Linton et al., 1999, 1998a, 1998b, 1997; Reid et al., 1998; Wood, 2004). Thus, branchial molecular responses to long-term, as opposed to acute ammonia exposure, still remain unexplored.

Most of the exposure studies to date have been done using optimal feeding or on fasted fish (Nawata et al., 2007; Wilkie et al., 2011; Wood, 2001; Zimmer et al., 2010). It has been shown for trout that feeding in itself can provide some protection against ammonia toxicity (Wicks and Randall, 2002b) and that feeding increases branchial mRNA expression of Rh genes, NHE-2 and H^+ -ATPase, as well as H^+ -ATPase activity in juvenile trout (Zimmer et al., 2010). In its natural habitat, food resources for salmon are more variable, and periods with low feed availability may occur (Saikkonen et al., 2011). Sub-optimal feeding can also be observed in aquaculture systems; however, its effects on the ability of Atlantic salmon parr to tolerate long-term ammonia exposure have not been studied so far.

We have previously reported that growth, welfare and smoltification of Atlantic salmon parr are not affected by long-term exposure to sublethal ammonia levels up to $1800 \mu\text{mol l}^{-1}$ TAN (Kolarevic et al., in press). In the present study, we hypothesized that the successful adaptation to elevated ammonia levels seen in Kolarevic et al. (in press) involved activation of several different ammonia detoxification strategies. Thus, we targeted several key biomarkers, such as glutamine levels in the brain and branchial excretion mechanisms through *Rhcg1*, *Rhcg2* and *H⁺-ATPase*, *UT* and *Aqp3a* in Atlantic salmon parr. We also investigated the effects of restricted feeding on Atlantic salmon parr tolerance to long-term sublethal environmental ammonia exposure.

2. Materials and methods

2.1. Experimental animals and design

The experimental animals and design were described in detail in Kolarevic et al. (in press). Briefly, during a 105-day long trial, Atlantic salmon with an average individual starting weight (SEM) of $17.1 (0.1) \text{ g}$, were divided into eight groups, each in triplicate tanks ($n=55$ fish per tank) and exposed to the combination of one of four chronic high environmental ammonia concentrations (HEA) and two feeding regimes: full feed strength with 20% of overfeeding, and 1/3 of full feed strength. Fish kept on the full feeding strength were exposed to average TAN values of: 4 ± 1 , 478 ± 37 , 809 ± 67 , or $1794 \pm 393 \mu\text{mol l}^{-1}$ (0 ± 0 , 0.7 ± 0.0 , 1.2 ± 0.0 , $2.5 \pm 0.1 \mu\text{mol l}^{-1}$ $\text{NH}_3\text{-N}$) while four groups with restricted feeding received 4 ± 1 , 465 ± 35 , 800 ± 65 , or $1704 \pm 378 \mu\text{mol l}^{-1}$ TAN (0 ± 0 , 0.6 ± 0.0 , 1.0 ± 0.0 , $2.3 \pm 0.1 \mu\text{mol l}^{-1}$ $\text{NH}_3\text{-N}$), respectively. Atlantic salmon exposed to $4 \mu\text{mol l}^{-1}$ TAN were used as control groups at both feeding levels. The average water temperature during the exposure was 12.11 ± 0.02 with a pH of 6.84 ± 0.02 ($\pm \text{SEM}$). The fish were kept at 24 h continuous light and fed with a commercial pelleted feed for 45 min every 8 h; this is a commonly used rearing protocol in Atlantic salmon smolt production (Stefansson et al., 1991).

To achieve the desired ammonia concentrations a stock solution of $(\text{NH}_4)_2\text{SO}_4$ was supplied from the mixing tank (Iwaki Unit; 21 g $(\text{NH}_4)_2\text{SO}_4 \text{ l}^{-1}$ fresh water) using a peristaltic pump (520DuN, Watson Marlow Bredel, Wilmington, USA) and marprene tubes with different diameter sizes. Water samples were collected and analysed daily for total ammonia nitrogen (TAN $\mu\text{mol l}^{-1}$) with an automated chemistry analyzer (Flow Solution IV, OI Analytical,

College Station, Texas, USA). In addition, water quality (pH, conductivity, oxygen) was monitored throughout the experiment as described in Kolarevic et al. (in press). Values for $\text{NH}_3\text{-N}$ were calculated using the temperature, conductivity, pH and TAN values for the day in question, using formulas from the American Fisheries Society website (Colt, 2011), based on Emerson et al. (1975).

At day 22 and 105 of exposure, five fish from each tank were terminally anesthetized with buffered MS 222 (metacaine, 0.1 g l^{-1} ; Argent Chemical Laboratories, Redmond, WA, USA). The fish were not fasted prior to sampling (Wood, 2001). Blood samples were collected from the caudal blood vessel using Vacuette® heparinized vacuum tubes (Greiner Bio-One, Kremsmünster, Austria) and centrifuged at $3000 \times g$ for 10 min (4°C). Plasma was stored at -80°C for subsequent analysis. Whole brain and gill samples were flash-frozen in liquid nitrogen and stored at -80°C for later FAA analysis and RNA extraction.

There were no observed ammonia exposure related mortalities during the study and the use of experimental animals for this trial was approved by the Norwegian Animal Research Authority. The “3R’s” principle (Russell and Burch, 1959) was applied and humane end points were established prior to start of the experiment.

2.2. Plasma ammonia [T_{amm}] and urea analyses

Equal volumes of plasma samples from within each tank were pooled together (plasma samples from five individual fish per tank), and subsequently used for urea and ammonia plasma analyses. For ammonia analysis, $160\text{ }\mu\text{l}$ plasma was deproteinized in $80\text{ }\mu\text{l}$ 8% perchloric acid on ice, and centrifuged at $15,000 \times g$ for 5 min (4°C). The supernatant was neutralized using 0.5 v/v saturated KHCO_3 and centrifuged at $7500 \times g$ for 30 s (4°C). Plasma total ammonia levels (T_{amm}) in the supernatant were analysed in duplicate using a commercial Ammonia Assay Kit (Sigma AA0100, Sigma–Aldrich, St. Louis, MO, USA). For urea analyses, $300\text{ }\mu\text{l}$ plasma was deproteinized in trichloroacetic acid (8% final concentration), stored overnight at 4°C and centrifuged at $3000 \times g$ for 10 min. Urea concentrations in blood plasma were measured in triplicate using the diacetyl monoxime method (Rahmatullah and Boyde, 1980) with appropriate adjustments according to Andersen et al. (2006). All spectrophotometric measurements were done on a Nicolet Evolution 500 UV–vis spectrophotometer (Thermo Electron Corporation, Marietta, Ohio, USA).

2.3. Brain free amino acids (FAAs)

For each tank, frozen brain samples from three individuals were weighed and equal amounts of tissues were pooled together. Free amino acids were extracted according to Davis (2002) and Oehme et al. (2010). Briefly, pooled samples (100 mg wet mass) were homogenized in 4 ml lithium citrate loading buffer (Biochrom, Cambridge, UK) with a porcelain mortar and pestle. After 1 h in a shaking water bath (SBK 25D, SalvisLab, Rotkreuz, Switzerland) at room temperature, the homogenate was centrifuged for 5 min ($10,000 \times g$, at room temperature; Eppendorf S415C, Hamburg, Germany). The resulting supernatant was deproteinized with sulfosalicylic acid (0.05 w/v) and filtered ($0.22\text{ }\mu\text{m}$ Ultrafree CL) (Davis, 2002; Oehme et al., 2010) before injection in a Biochrom 30 amino acid analyzer (Biochrom Ltd., UK).

2.4. RNA isolation and cDNA synthesis

RNA was isolated from frozen gills ($n=8\text{--}15$ per treatment) using Precellyser 24 (Bertin Technologies, France), Trizol reagents and the Micro to Midi Kit (Invitrogen, CA, USA). All samples were DNase treated (DNase1, Invitrogen). The quality of the RNA was assessed spectrophotometrically (NanoDrop Technologies, DE,

Table 1

Primers used for real time PCR expression analysis of Atlantic salmon (*Salmo salar*) parr genes.

| Gene | Accession number | Orientation | Sequence (5′ – 3′) |
|----------|------------------|-------------|-------------------------|
| Rhcg1 | DQ431244 | Forward | CATCCTCAGCCTCATACATGC |
| Rhcg1 | DQ431244 | Reverse | TGAATGACAGACGGAGCCAATC |
| Rhcg2 | AY619986 | Forward | CCTCTTCGGAGTCTTCATC |
| Rhcg2 | AY619986 | Reverse | CTATGTCGCTGGTGATGTTG |
| H-ATPase | NM001141746 | Forward | TACGGCACTGAGGGTTGATG |
| H-ATPase | NM001141746 | Reverse | TTACCTGACGGCACCAAGAG |
| UT | EF6888013 | Forward | GTATAGGCCAGGTGTATGGG |
| UT | EF6888013 | Reverse | GATCGCCTCAATGGAGCTG |
| Aqp3a | Ssa. 27124 | Forward | GTGACAGGAAGAGCCAGGAG |
| Aqp3a | Ssa. 27124 | Reverse | TGAGGCTGAGCTTAGGGGTA |
| Ef1a | DQ834870 | Forward | CACCACCGGCATCTGATCTACAA |
| Ef1a | DQ834870 | Reverse | TCAGCAGCTCTTCTCGAACTTC |

USA) and $0.5\text{ }\mu\text{g}$ RNA was reverse transcribed to cDNA using oligo (dT) and random hexamer primers and the Taqman Gold RT-PCR kit (Applied Biosystems, CA, USA). The cDNA synthesis was performed with 10 min primer incubation at 25°C , 1 h RT step at 48°C and 5 min RT inactivation at 95°C . All reactions were performed in accordance with the manufacturer’s protocol.

2.5. Real-time quantitative RT-PCR

Primers for transcription analysis (Table 1) were based on known Atlantic salmon sequences or on conserved regions of known teleost sequences paralogues in GenBank (Benson et al., 2000) using BLAST similarity search (Altschul et al., 1997). Primers were designed using the Vector NTI Advance 10 (Invitrogen) and NetPrimer (PREMIER Biosoft, CA, USA) software. All PCR products were cloned using pGEM T-easy (Promega, WI, USA) and sequenced with Big Dye Terminator chemistry and the ABI 3730 automated sequencer (Applied Biosystems, CA, USA). The obtained Atlantic salmon clones were analyzed and verified by BLAST. RT-PCR was conducted using the Light cycler 480 and SYBR Green chemistry (Roche, Switzerland) at the following thermal cycling conditions: 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, $60 \pm 1^\circ\text{C}$ for 15 s and 72°C for 15 s. Further, specificity was assessed by the melting curves, determined post PCR (95°C for 15 s, 60°C for 1 min and 97°C continuous). To determine the efficiency of target genes and reference gene (elongation factor 1a), the standard curve method was used. Relative target gene mRNA was normalized to relative ef1a mRNA levels for all samples, as recommended by Olsvik et al. (2005).

2.6. Statistical analyses

A multivariate analysis of variance (MANOVA) was used to examine the influence of ammonia treatment and feed regime and their interaction on plasma urea and ammonia levels and brain FAAs. For comparison of ammonia responses at different time points, in cases when one-way analysis of variance (ANOVA) was significant, multiple comparisons tests (Student’s t-test for each pair) were performed to establish difference between control and other treatments. The statistical analyses were carried out with JMP 9.0 software (SAS 2010, SAS Institute Inc, Cary, USA).

The transcription ratios were analyzed using the Relative Expression Software Tool (REST) (Pfaffl et al., 2002). Real time qPCR results were tested for significance by the Pair Wise Fixed Reallocation Randomization Test© (Pfaffl et al., 2002). All data are expressed as means \pm standard error of mean (SEM), and difference between treatments were judged significant if $p \leq 0.05$.

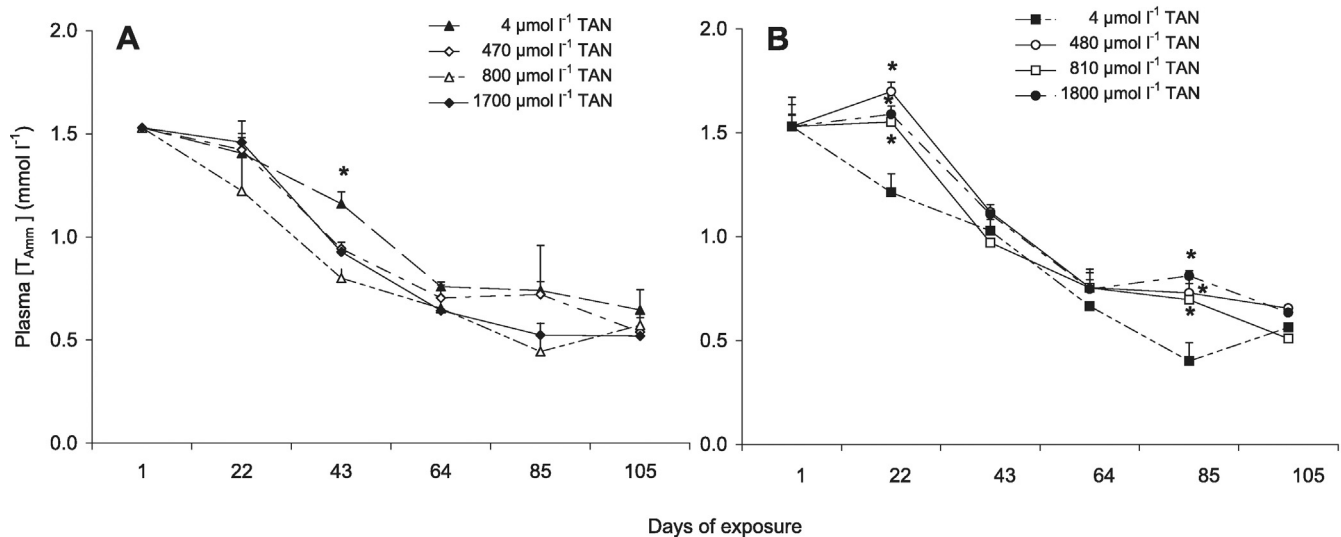


Fig. 1. Plasma total ammonia concentration [T_{Amm}] in fish of the groups with restricted feeding (A) and in full fed groups (B) at day 1, 22, 43, 64, 85 and 105 during long-term ammonia exposure. Asterisk indicates significant difference in plasma ammonia between control and exposed treatments. All values are given as means + SEM ($n = 3$).

3. Results

3.1. Plasma ammonia and urea levels

A significant two-way interaction between the factor feeding ration and the factor HEA was noted after 22 and 43 days of exposure with respect to plasma [T_{Amm}] ($p < 0.05$) (Fig. 1). Plasma ammonia concentrations measured on day 22 were significantly higher than at the end of exposure study (day 105) for all treatments ($p < 0.05$). For the groups with restricted feeding, ammonia exposure did not cause a significant increase in plasma [T_{Amm}] (Fig. 1a). Instead, plasma [T_{Amm}] significantly decreased between day 22 and 64 of exposure ($p < 0.05$) in all treatments, and then tended to stabilize or decrease slightly until the end. In contrast, plasma [T_{Amm}] increased until day 22 in the full fed groups exposed to HEA (Fig. 1b), showing significantly higher levels than the control group fish, which were not exposed to ammonia. Although plasma [T_{Amm}] decreased towards the end of experiment, at day 85 it was significantly higher in the full fed fish exposed to ammonia ($p < 0.05$), in comparison to the control group fish. This difference, however, was not observed at the end of the experiment (Fig. 1b).

Throughout the HEA exposure, plasma urea levels showed a dose-dependent decline with ammonia exposure concentration regardless of the feeding regime (Fig. 2a, b). Significant differences between plasma urea concentrations between day 22 and day 105 of exposure were observed for full fed fish exposed to 1800 μmol l⁻¹ TAN and salmon at restricted feeding regime exposed to 470 μmol l⁻¹ TAN ($p < 0.05$). Tendency for decrease in plasma urea concentration over time ($p < 0.1$) was noted when restricted feeding regime was combined with 4, 800 and 1700 μmol l⁻¹ TAN exposure and at full feeding levels and exposure to 480 μmol l⁻¹ TAN. There was no significant interaction between the ammonia exposure factor and feeding ration factor, on plasma urea levels at any time point. The lowest concentrations of plasma urea were noted for the groups exposed to 1700 and 1800 μmol l⁻¹ TAN, while plasma urea levels remained the highest in the control groups at all times. Significantly higher plasma urea levels were found in fish of the control groups at the day 105, while the feeding regime did not significantly affect this plasma parameter.

3.2. Brain free amino acids (FAAs)

Between 57 and 63% of the total brain free amino acid pool (without taurine) consisted of six FAAs (Tables 2 and 3) in all examined experimental groups. Remaining FAAs detected in the brain of Atlantic salmon parr were not significantly different at investigated time points. The concentrations of major FAAs were not significantly affected by interactions between ammonia and feeding level. Among the full fed groups, a significantly higher concentration of Gln was found in Atlantic salmon brain tissue, when exposed to 1800 μmol l⁻¹ TAN at day 22, while at the same time there was a tendency ($p = 0.09$) for a decrease in Glu levels in this group. Furthermore, aspartic acid (Asp) concentrations were significantly lower at 480 and 1800 μmol l⁻¹ TAN and lysine (Lys) at 1800 μmol l⁻¹ TAN, compared to the corresponding control fish. A tendency for increases in Gln and threonine (Thr) levels was observed in Atlantic salmon exposed to 1700 μmol l⁻¹ TAN, and restricted feeding regime ($p < 0.1$) (Table 2). Significantly higher brain Gln concentrations were measured at day 105, but only in individuals exposed to the highest ammonia concentrations (1700 and 1800 μmol l⁻¹ TAN), regardless of feeding regime (Table 3). The combination of 1700 μmol l⁻¹ TAN exposure and restricted feeding, also caused an increase in brain alanine (Ala) concentration ($p < 0.05$) at the end of the exposure period.

3.3. Branchial gene transcription

Ammonia exposure significantly stimulated the branchial transcription of genes encoding Rh glycoproteins, but the response was modulated both by exposure concentration, duration of exposure and feeding regime (Fig. 3). In fish subjected to the restricted feeding regime, only exposure to 1700 μmol l⁻¹ TAN caused a two-fold up-regulation of *Rhcg1* at day 22 compared to the control group. A concentration-dependent increase in the transcription of *Rhcg1* was observed at day 105 compared to the control group with two-, three- and four-fold higher expression levels in fish exposed to 470, 800 and 1700 μmol l⁻¹ TAN, respectively (Fig. 3a). In the full fed groups, branchial mRNA expression of *Rhcg1* was two- to four-fold higher in the groups exposed to HEA at day 22 (Fig. 3b) compared to the control group. At day 105, the relative *Rhcg1* transcription decreased in the group exposed to 480 μmol l⁻¹ TAN, slightly

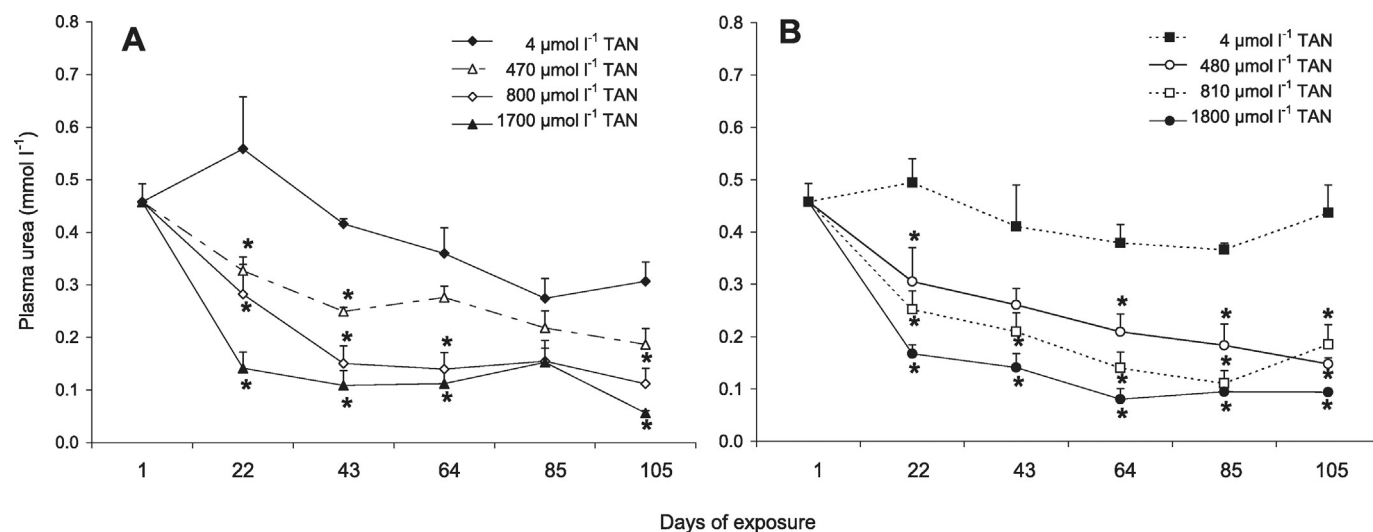


Fig. 2. Plasma urea concentration in fish of the groups with the restricted feeding (A) and in the full fed groups (B) at day 1, 22, 43, 64, 85 and 105 during long-term ammonia exposure. Asterisk indicates significant difference in plasma urea between control group and groups exposed to environmental ammonia among full fed and groups with restricted feeding. All values are given as means \pm SEM ($n = 3$).

Table 2
Concentration of major free amino acids (FAA) in the brain (nmol mg⁻¹ dry matter) of Atlantic salmon exposed to four ammonia concentration and two feeding regimes (restricted feeding and full feeding) after 22 days. Values are given as means \pm SEM ($n = 3$). Capital letters denote significant differences (Student's *t*-test for each pair; $P < 0.05$) between full feed treatments.

| FAA | Ammonia treatments | | | | | | | |
|-----|--------------------------------|------------------------------|------------------------------|-------------------------------|------------------------------|-------------------------------|-------------------------------|-------------------------------|
| | Groups with restricted feeding | | | | Groups with full feeding | | | |
| | 4 μmol l ⁻¹ TAN | 470 μmol l ⁻¹ TAN | 800 μmol l ⁻¹ TAN | 1700 μmol l ⁻¹ TAN | 4 μmol l ⁻¹ TAN | 480 μmol l ⁻¹ TAN | 810 μmol l ⁻¹ TAN | 1800 μmol l ⁻¹ TAN |
| Gln | 6.11 \pm 0.58 | 7.29 \pm 0.15 | 6.41 \pm 0.56 | 8.97 \pm 1.26 | 6.83 \pm 0.06 ^B | 7.16 \pm 0.50 ^{AB} | 7.15 \pm 0.05 ^{AB} | 8.91 \pm 0.62 ^A |
| Glu | 6.10 \pm 0.27 | 6.49 \pm 0.13 | 5.63 \pm 0.20 | 6.16 \pm 0.39 | 6.57 \pm 0.11 | 6.09 \pm 0.28 | 6.46 \pm 0.11 | 5.76 \pm 0.27 |
| Asp | 1.50 \pm 0.10 | 1.52 \pm 0.03 | 1.32 \pm 0.03 | 1.45 \pm 0.17 | 1.62 \pm 0.01 ^A | 1.42 \pm 0.08 ^{AB} | 1.49 \pm 0.04 ^{AB} | 1.34 \pm 0.06 ^B |
| Thr | 0.79 \pm 0.07 | 0.90 \pm 0.06 | 0.83 \pm 0.09 | 1.04 \pm 0.03 | 0.90 \pm 0.08 | 0.90 \pm 0.07 | 0.87 \pm 0.08 | 0.96 \pm 0.03 |
| Lys | 0.35 \pm 0.04 | 0.38 \pm 0.03 | 0.32 \pm 0.06 | 0.33 \pm 0.10 | 0.43 \pm 0.02 ^A | 0.36 \pm 0.05 ^{AB} | 0.35 \pm 0.04 ^{AB} | 0.27 \pm 0.01 ^B |
| Ala | 0.82 \pm 0.06 | 0.80 \pm 0.17 | 0.81 \pm 0.24 | 0.91 \pm 0.25 | 0.81 \pm 0.06 | 1.02 \pm 0.05 | 0.93 \pm 0.04 | 0.84 \pm 0.20 |

increased in the group exposed to 810 μmol l⁻¹ TAN and remained the same in fish exposed to 1800 μmol l⁻¹ TAN, when compared to day 22. However, in fish of all three HEA groups, the relative mRNA levels were still significantly higher compared to the control group that was not exposed to ammonia ($p < 0.05$).

Levels of *Rhcg2* were two-fold higher in the fish exposed to 480 μmol l⁻¹ TAN and varied within one order of magnitude for other experimental groups at day 22 (Fig. 3c, d). A two- to three-fold increase in *Rhcg2* transcription was noted at the end of the experiment, regardless of the feeding regime. However, in the group exposed to ~800 μmol l⁻¹ TAN, no effect on levels of *Rhcg2* mRNA was observed during the whole experiment (Fig. 3c, d).

Table 3
Concentration of major free amino acids (FAA) in the brain (nmol mg⁻¹ dry matter) of Atlantic salmon exposed to four ammonia concentration and two feeding regimes (restricted feeding and full feeding) after 105 days. Values are given as means \pm SEM ($n = 3$). Capital letters denote significant differences (Student's *t*-test for each pair; $P < 0.05$) between full feed treatments and small letters denote significant differences (Student's *t*-test for each pair; $P < 0.05$) between groups with restricted feeding regime. Asterisk denote significant difference (Student's *t*-test, $P < 0.05$) between feeding regimes exposed to the same ammonia concentration.

| FAA | Ammonia treatments | | | | | | | |
|-----|--------------------------------|-------------------------------|-------------------------------|-------------------------------|------------------------------|-------------------------------|-------------------------------|-------------------------------|
| | Groups with restricted feeding | | | | Groups with full feeding | | | |
| | 4 μmol l ⁻¹ TAN | 470 μmol l ⁻¹ TAN | 800 μmol l ⁻¹ TAN | 1700 μmol l ⁻¹ TAN | 4 μmol l ⁻¹ TAN | 480 μmol l ⁻¹ TAN | 810 μmol l ⁻¹ TAN | 1800 μmol l ⁻¹ TAN |
| Gln | 6.06 \pm 0.41 ^b | 7.04 \pm 0.31 ^b | 7.76 \pm 0.3 ^{ab7} | 9.25 \pm 0.58 ^a | 6.05 \pm 0.18 ^b | 6.92 \pm 0.42 ^{AB} | 6.71 \pm 0.33 ^{AB} | 9.05 \pm 0.87 ^A |
| Glu | 7.03 \pm 0.21 | 6.79 \pm 0.23 | 7.25 \pm 0.16 | 6.72 \pm 0.40 | 6.75 \pm 0.41 | 6.73 \pm 0.29 | 6.38 \pm 0.19 [*] | 6.63 \pm 0.27 |
| Asp | 1.67 \pm 0.10 | 1.68 \pm 0.02 | 1.63 \pm 0.03 | 1.62 \pm 0.04 | 1.64 \pm 0.08 | 1.53 \pm 0.04 [*] | 1.41 \pm 0.07 [*] | 1.54 \pm 0.15 |
| Thr | 0.88 \pm 0.08 | 0.96 \pm 0.00 | 1.11 \pm 0.13 | 1.06 \pm 0.07 | 0.78 \pm 0.09 | 0.97 \pm 0.12 | 0.98 \pm 0.11 | 1.13 \pm 0.10 |
| Lys | 0.28 \pm 0.04 | 0.34 \pm 0.02 | 0.31 \pm 0.02 | 0.28 \pm 0.02 | 0.35 \pm 0.03 | 0.27 \pm 0.01 | 0.22 \pm 0.05 | 0.27 \pm 0.03 |
| Ala | 0.78 \pm 0.03 ^{ab} | 0.70 \pm 0.07 ^{ab} | 0.62 \pm 0.02 ^b | 0.95 \pm 0.10 ^a | 0.70 \pm 0.07 | 0.57 \pm 0.08 | 0.85 \pm 0.01 [*] | 0.73 \pm 0.13 |

The transcription of branchial *H⁺-ATPase*, the product of which is important in maintaining a proper acidification of the gill boundary layer, was significantly lower in nearly all HEA groups at 22 days of exposure when compared to the control groups (Fig. 3e, f). A similar pattern of expression was observed for *H⁺-ATPase* at both feeding regimes at day 105 (Fig. 3e, f). Groups exposed to the highest levels of environmental ammonia, 1700 and 1800 μmol l⁻¹ TAN, had two-fold higher levels of *H⁺-ATPase* compared to the control groups (4 μmol l⁻¹ TAN), while this gene remained transcriptionally down-regulated only in groups exposed to ~800 μmol l⁻¹ TAN.

The potential role of branchial transporters of urea and ammonia during long-term ammonia exposure, were examined by analysing

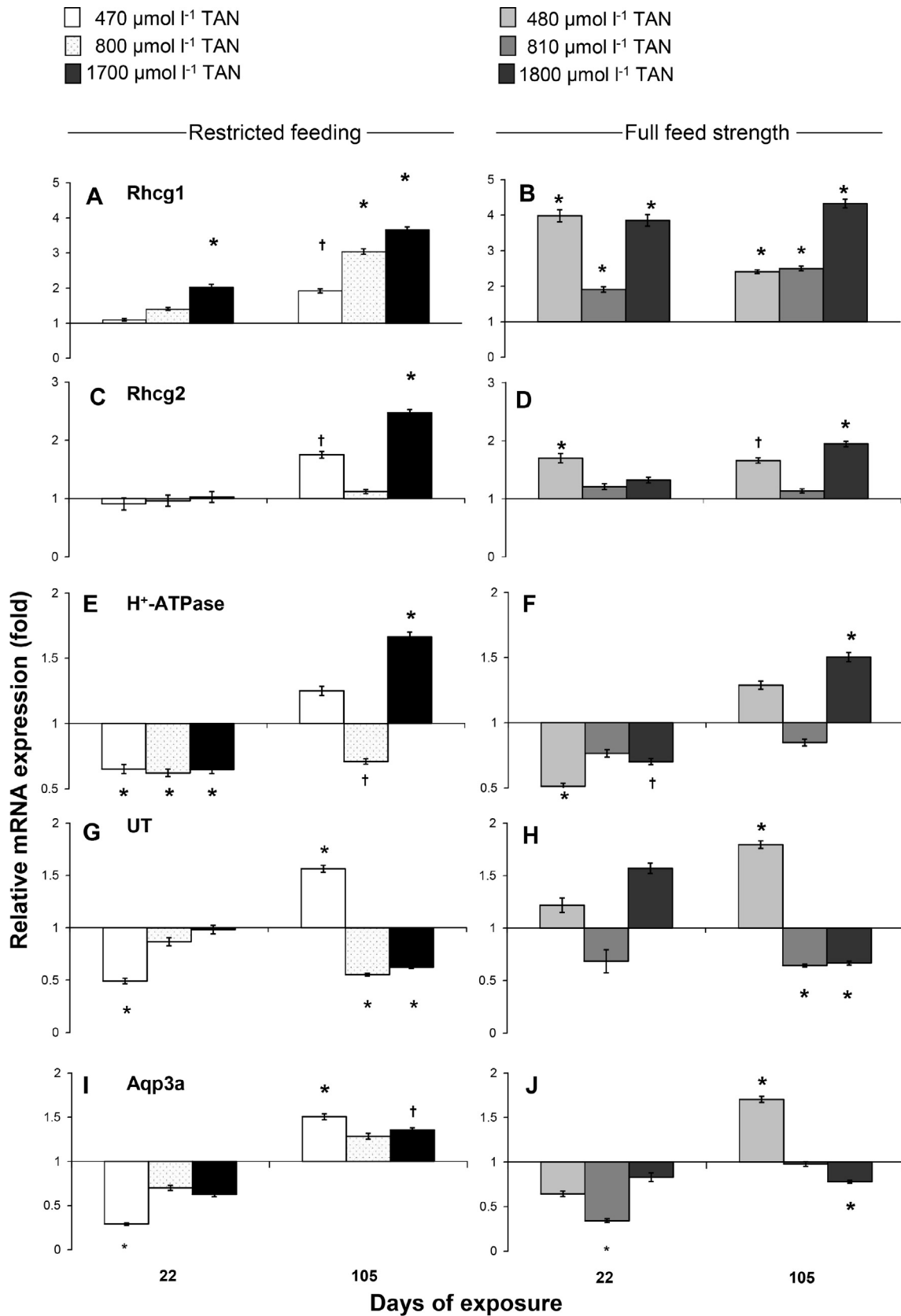


Fig. 3. Branchial mRNA expression levels of *Rhcg1*, *Rhcg2*, *H⁺-ATPase*, *UT* and *Aqp3a* in Atlantic salmon parr with restricted feeding (A, C, E, G, I) and full fed fish (B, D, F, H, J) after 22 and 105 days of exposure to sub lethal environmental ammonia. The mRNA expression level of the control groups has been set to 1 and is indicated with the x-axis; all other expression data are shown relative to this control group. Expression levels <1 indicate down-regulated genes and levels >1 show up-regulated genes compared to the control group expression levels (value of 1). Significant differences between groups exposed to ammonia and their corresponding control groups are denoted with asterisk ($p < 0.05$) or dagger ($p < 0.1$). Data are means \pm SEM ($n = 8-15$).

the transcription of *UT* and *Aqp3a*. Relative *UT* levels showed a two-fold increase at the lowest exposure concentrations of 470–480 $\mu\text{mol l}^{-1}$ TAN compared to the control groups, regardless of feeding regime, while *UT* was significantly transcriptionally down-regulated in the other HEA groups (Fig. 3g, h) at the end of the experiment. *Aqp3a* mRNA levels were down-regulated at day 22 and only significantly increased ($p < 0.05$) at day 105 in the groups treated with 470–480 $\mu\text{mol l}^{-1}$ TAN when compared to the control groups (Fig. 3i, j).

4. Discussion

This study suggests that Atlantic salmon parr adapts to long-term sublethal ammonia exposure by a combination of ammonia detoxification in the brain and an increase in the capacity for branchial excretion of ammonia and urea, provided that the transcriptional patterns demonstrated here are reflected in similar translational changes. In particular, *Rhcg1* plays an important role in ammonia excretion in Atlantic salmon parr during long-term exposure to HEA, contrary to the results of acute exposure studies (Nawata et al., 2007; Tsui et al., 2009; Wood and Nawata, 2011) in other salmonids. In addition, we show that plasma urea levels and transcription of genes related to branchial urea handling, are affected by long-term ammonia exposure. Furthermore, Gln synthesis is an ammonia detoxifying strategy utilised in the brain of Atlantic salmon parr. The major finding from the feeding ration factor, was that restricted feeding acted to postpone the increase in the transcription of genes encoding several proteins involved in the process of ammonia excretion and the Gln synthesis in the brain, observed in the full fed Atlantic salmon parr exposed to the HEA.

Monitoring of the plasma ammonia levels revealed that Atlantic salmon parr successfully adapt to HEA. In full fed fish, the time spent before plasma $[T_{\text{amm}}]$ was normalised was more than 3 weeks, while fish fed a restricted diet did not show any increased levels after exposure to HEA. Increases in plasma $[T_{\text{amm}}]$ occur in response to feeding (Buckling and Wood, 2008; Wicks and Randall, 2002a,b; Zimmer et al., 2010), and exposure to elevated environmental ammonia concentrations (Rasmussen and Korsgaard, 1998; Schram et al., 2010; Vedel et al., 1998; Zimmer et al., 2010). Among ammonia-exposed fish, the full feeding regime additionally increased plasma ammonia after 22 days, showing the additive effect of feeding on plasma $[T_{\text{amm}}]$, as observed in juvenile rainbow trout (Zimmer et al., 2010). The $[T_{\text{amm}}]$ observed in the present study is in the upper range reported for salmonids, mostly obtained from sampling of fasting fish (Wood, 1993; 2001). However, Wicks and Randall (2002b) found that in rainbow trout of 45 g ind $^{-1}$, plasma ammonia peaked at 994 $\mu\text{mol l}^{-1}$ after feeding, which is comparable to day 43–64 in the present study (Fig. 1). The cause for the decrease in plasma $[T_{\text{amm}}]$ over time observed in the present study is not clear, but similar trends have been observed previously. During rearing of African catfish (*Clarias gariepinus*) larvae and juveniles, the whole-body $[T_{\text{amm}}]$ declined slightly, but significantly, over time and a body weight range of 2.5–97 mg ind $^{-1}$ (Terjesen, 1995). Furthermore, in a study on turbot (*Scophthalmus maximus*) the plasma $[T_{\text{amm}}]$ declined significantly during a 58 day long experiment when the turbot more than doubled their individual weight (Ruyet et al., 2003). Based on the molecular responses of genes encoding ammonia transporting proteins described here we hypothesize that the observed decrease in plasma $[T_{\text{amm}}]$ in Atlantic salmon parr and the successful adaptation to elevated ammonia levels involve activation of branchial excretion mechanisms.

The most pronounced branchial molecular response to the long-term sublethal ammonia exposure was the up-regulation of *Rhcg1* mRNA observed at all investigated HEA. In the full fed groups we did not observe a similar concentration-response profile as in the

fish fed a restricted diet, but the strong transcriptional activation argues that *Rhcg1* is a profound biomarker for long-term ammonia exposure. Considering the increase in plasma $[T_{\text{amm}}]$ in full fed fish after 22 days of exposure, it remains uncertain whether the transcriptional *Rhcg1* up-regulation was established as an immediate response to sublethal ammonia levels. Branchial transcription of *Rhcg1* has not been responsive to high external ammonia exposure in several acute studies (Braun et al., 2009a; Moreira-Silva et al., 2010; Nakada et al., 2007; Tsui et al., 2009). However, a recent study showed that this gene was transcriptionally up-regulated in delta smelt (*Hypomesus transpacificus*) after a short-term exposure to HEA (Connon et al., 2011). Available information on the effects of ammonia on *Rhcg1* mRNA expression in rainbow trout is complex. Early studies indicated that *Rhcg1* is not responsive to HEA (Nawata et al., 2007), but ammonia infusion increased mRNA expression of *Rhcg1* in rainbow trout gills (Nawata and Wood, 2009). Contrary to this, Wood and Nawata (2011) showed a progressive transcriptional down-regulation of *Rhcg1* during 24 h exposure to HEA in rainbow trout gills. In all three acute exposure studies, the experimental animals were fasted one week before the start of the experiment and the highest plasma $[T_{\text{amm}}]$ were recorded after ammonia infusion, when mRNA levels of *Rhcg1* increased. We hypothesise that in the present study, the high HEA and the combination of full ration feeding and exposure to HEA, triggers *Rhcg1* responses. Furthermore, we argue that this Rh glycoprotein is a key branchial transporter ensuring elimination of excess ammonia during long-term exposure to HEA in Atlantic salmon. Our results also suggest a long term transcriptional activation of *Rhcg1* with possible turnover of this transporter.

The results of this study indicate that *Rhcg2* and H^+ -ATPase are also involved in ammonia removal during long-term exposure, but apparently limited to certain HEA in Atlantic salmon. The branchial *Rhcg2* transcriptional response observed in the present study was concentration dependent and mostly evident at the end of the experiment. In addition, restricted feeding apparently delayed the *Rhcg2* mRNA increase, seen for *Rhcg1*. Up-regulation of branchial *Rhcg2* and H^+ -ATPase expression plays a key role during short-term exposure to HEA in rainbow trout (Nawata et al., 2007; Tsui et al., 2009; Wood and Nawata, 2011; Zimmer et al., 2010). Apically located H^+ -ATPase, together with NHE and hydration of CO_2 , are thought to provide the acidification of the gill boundary layer (Ivanis et al., 2008; Lin et al., 1994; Wilkie, 2002). In the current study, H^+ -ATPase transcription decreased after 22 days of exposure compared to the control groups, and became up-regulated at the end of experiment, but this was limited to the highest and the lowest HEA. This observation coincided with the up-regulation of *Rhcg2* mRNA at those exposure concentrations. Temporal correlation of *Rhcg2* and H^+ -ATPase mRNA upregulation was found in rainbow trout gills and coincided with the recovery of ammonia excretion during HEA (Nawata et al., 2007). In the Asian weatherloach (*Misgurnus anguillicaudatus*), H^+ -ATPase tissue activity and protein levels did not increase in response to HEA. However, the use of a V-ATPase inhibitor, bafilomycin, decreased the ammonia flux, resulting in elevation of plasma $[T_{\text{amm}}]$, and indicating the importance of this protein for ammonia excretion, although without an observed increase in its branchial expression level (Moreira-Silva et al., 2010). On the other hand, an in situ study of mitochondria-rich cells in the larvae of Japanese medaka (*Oryzias latipes*) showed that the ammonia excretion through *Rhcg1* was dependent on H^+ excretion by NHE-3 while being insensitive to bafilomycin treatment (Wu et al., 2010). Further studies are necessary to determine the mechanisms of the gill boundary layer acidification in Atlantic salmon in fresh water.

The response of the majority of investigated branchial genes in the present study resembled the biphasic pattern seen in delta

smelt after a short-term exposure to HEA (Connon et al., 2011). In that study, the lowest mRNA expression levels for a number of genes were found at the medium or low-observed effects concentrations. The lowest mRNA expression levels for nearly all investigated genes in our study were found in fish exposed to the medium HEA of $\sim 800 \mu\text{mol l}^{-1}$ TAN ($P_{\text{NH}_3} \sim 20 \mu\text{Torr}$). In fact, a similar partial pressure of ammonia ($P_{\text{NH}_3} \sim 23 \mu\text{Torr}$) promoted growth of rainbow trout after 70 days of exposure (Wood, 2004). Although we did not observe any ammonia-related mortalities at the tested ammonia concentrations, we speculate that the biphasic response observed can be indicative of two exposure limits that could be potentially beneficial or detrimental for long-term fish welfare, respectively. Further studies are necessary in order to explore this hypothesis.

A progressive decrease in plasma urea levels was observed throughout the experiment. This trend depended on the HEA but was not significantly affected by feeding regime. After three weeks of exposure the largest decrease in plasma urea was caused by the combination of high HEA ($1800 \mu\text{mol l}^{-1}$ TAN) and full feeding ration, and it coincided with the observed tendency for higher transcription of *UT* in this group. A simultaneous decrease in plasma urea, and increase in branchial *UT* transcription, was observed after a short-term exposure of rainbow trout to HEA and this was tied to an increase in urea excretion (Wood and Nawata, 2011). Furthermore, Foss et al. (2004) found that Atlantic cod (*Gadus morhua*) responded to increasing HEA concentrations (96 day chronic exposure) with a significant decrease in plasma urea. An HEA-increased mRNA expression of *UT* was also found in the gills of adult zebrafish, with a transient increase in urea excretion (Braun et al., 2009a). Transcription of *UT* was also responsive to low HEA as seen in the present study after exposure of Atlantic salmon parr to $\sim 480 \mu\text{mol l}^{-1}$ TAN. In addition, we observed increased transcription levels of *Aqp3* which coincided with transcriptional *UT* up-regulation at $\sim 480 \mu\text{mol l}^{-1}$ TAN. Several recent reports indicate the existence of additional pathways for urea excretion, other than through branchial *UT* (Braun et al., 2009b; McDonald and Wood, 2004; Weihrach et al., 2009; Wilkie, 2002). *Aqp3* is a water channel that is also permeable to urea and glycerol (MacIver et al., 2009; Tingaud-Sequeira et al., 2009). In the present study, we show for the first time, that mRNA expression of *Aqp3a*, encoding one member of the *Aqp* family, is responsive to ammonia exposure in fish. A similar response in *Aqp3a* and *UT* transcription to HEA might indicate a functional coupling of the protein gene products in urea excretion. Further investigations are necessary in order to determine the localization of *Aqp3* and *UT* in the gills of Atlantic salmon parr.

The present study demonstrates that during chronic exposure, ammonia detoxification takes place in the brain of Atlantic salmon. Increased Gln levels were found in response to high HEA ($1800 \mu\text{mol l}^{-1}$ TAN), and full feeding, after 22 days of exposure, while restricted feeding had a delayed effect on the increase in brain Gln levels, evident only after 105 days of exposure to $1700 \mu\text{mol l}^{-1}$ TAN. Increases in brain GSase activity and Gln concentration have been reported for a number of fish species in response to ammonia exposure (Hegazi et al., 2010; Peng et al., 1998; Wicks and Randall, 2002a). Gln synthesis in the brain can be followed by a decrease in Glu levels (Hegazi et al., 2010), but in some cases the concentrations of Glu remain unchanged (Peng et al., 1998), as was observed in this study. This result suggests an efficient replenishment of Glu used for Gln synthesis in the brain of Atlantic salmon after a long-term exposure to HEA. Significant inhibition ($\sim 75\%$) of brain GSase activity in rainbow trout, however, did not change brain ammonia or Glu levels, indicating that an alternative pathway exist in this tissue to maintain acceptable levels of these compounds (Sanderson et al., 2010). A decrease in Asp brain concentration in response to ammonia exposure was observed in the present study,

as seen earlier for rainbow trout (Sanderson et al., 2010), Nile tilapia (*Oreochromis niloticus*) (Hegazi et al., 2010) and giant mudskipper (*Periophthalmodon schlosseri*) (Peng et al., 1998). Asp can serve as a precursor for synthesis of Glu via transaminase reaction (Sanderson et al., 2010) and prevent depletion of Glu levels in the brain. In addition, we observed an increase in Ala brain concentration at the restricted feeding level. Increases in the activity of the cytosolic alanine aminotransferase (c-ALT) was found to coincide with an increase in Ala level in the brain of Nile tilapia (Hegazi et al., 2010). Ala production through partial amino acid catabolism has been proposed as an efficient way of minimizing ammonia accumulation in the giant mudskipper (Ip et al., 2001b). Contrary to the energetically expensive Gln synthesis, transamination of Glu and pyruvate is followed by a favourable ATP yield (Ip et al., 2001b) and as such could be beneficial during suboptimal feeding.

In conclusion, this is the first report on molecular responses of genes encoding ammonia and urea transporting proteins during long-term exposure to HEA. In Atlantic salmon parr, Rhcg1 seems to play an important role during long-term ammonia excretion, in contrast to the lack of an immediate Rhcg1 response during short-term acute ammonia exposure reported previously. Furthermore, urea excretion via *UT* and *Aqp3* is stimulated by the long-term exposure to ammonia, as well as Gln formation in the brain tissue of Atlantic salmon. Further investigations are necessary to determine if the underlying defense mechanisms against long-term ammonia exposure reported here, are widely spread among freshwater teleosts.

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